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SEROPREVALENCE OF *TRYPANOSOMA CRUZI* IN RACCOONS FROM TENNESSEE

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ABSTRACT: *Trypanosoma cruzi* is the etiologic agent of Chagas' disease. Autochthonous human and canine transmission of *T. cruzi* has been documented in Tennessee, but little is known about its ecology, including the prevalence of *T. cruzi* among wildlife in Tennessee. Serum samples from 706 raccoons (*Procyon lotor*) from 10 counties in the Ridge and Valley and Blue Ridge Mountains ecoregions of eastern Tennessee were tested for antibodies reactive with *T. cruzi* using the indirect fluorescent antibody assay. Two hundred six (29.2%) samples were seropositive, with 9 counties yielding positive samples (range 14.6–63.6%). Significantly more raccoons from rural habitats (35.1%) were found positive for *T. cruzi* exposure than were those from suburban habitats (23.1%, $P < 0.001$). Land cover class was not associated with seropositivity status ($P = 0.441$), even though deciduous forest was the most common site from where raccoons were trapped and the most common site of positive raccoons in rural areas (42%). Interestingly, age was positively associated with seropositivity. Raccoons older than 1 yr (adults) were 40.1% seropositive compared to 12.2% of those less than 1 yr (juveniles; $P < 0.001$). Female adults were significantly more likely to be exposed to *T. cruzi* than were male adult raccoons ($P < 0.001$). No significant seroprevalence difference was seen among male and female juveniles. This study contributes to understanding the dynamics of *T. cruzi* exposure within raccoon populations in Tennessee. The importance of habitat (rural vs. suburban) and microhabitat (dens) in risk of exposure to these populations is also discussed.

Trypanosoma cruzi is the causative agent of Chagas' disease, which infects 8–11 million people in Latin America (CDC, 2009). In the United States, *T. cruzi* is endemic in some wildlife populations, but autochthonous human infections are rarely documented. Of the 7 cases that have been reported since 1955, 4 were in children in Texas (1955, 1983, and 2006), 1 in a 56-yr-old woman in California (1982), 1 in an 18-mo-old child in Tennessee (1998), and 1 in a 74-yr-old woman in Louisiana (2006) (Herwaldt et al., 2000; Dorn et al., 2007; Kjos et al., 2008). *Trypanosoma cruzi* infection in animals in the United States is well established and, recently, serological methods have been used to detect *T. cruzi* infections in raccoons (*Procyon lotor*) from Arizona, Florida, Georgia, South Carolina, Missouri, and Virginia (Yabsley and Noblet, 2002; Hancock et al., 2005; Brown et al., 2009); opossums (*Didelphis virginiana*) from Georgia, Florida, and Virginia (Brown et al., 2009); wild canids (Canidae) from South Carolina, Georgia, and Virginia (Rosypal et al., 2007; Brown et al., 2009); bobcats (*Felis rufus*) from Georgia (Brown et al., 2009); striped skunks (*Mephitis mephitis*) from Arizona and Georgia (Brown et al., 2009); a ringtail (*Bassariscus astutus*) from Arizona (Brown et al., 2009); and lemurs (*Lemur catta*, *Eulemur macaco flavifrons*, *Varecia variegata variegate*) in Georgia (Hall et al., 2007). Although an autochthonous human case of *T. cruzi* infection has occurred in Tennessee, no comprehensive study has been conducted to investigate the ecology or transmission risk of *T. cruzi* to humans or other animals in Tennessee. Ecologically, the transmission of particular strains of *T. cruzi* has been shown to differ with respect to habitat, such that *T. cruzi* strain I is predominantly found in sylvatic settings, while *T. cruzi* II is found in domestic settings (Devillers et al., 2008). In Brazil, certain landscapes, mainly forested areas, are associated with *T. cruzi* transmission (Herrera et al., 2007). To determine whether landcover can influence *T. cruzi* exposure in our study area, we conducted a spatial analysis of our samples with respect to landscape. To improve understanding of the prevalence and infection risk of *T. cruzi* in Tennessee, the seroprevalence of *T. cruzi* in raccoons from 10 eastern counties was examined using the indirect fluorescent antibody (IFA) assay.

MATERIALS AND METHODS

Sample collection

From 2005 to 2007, the United States Department of Agriculture, Animal and Plant Health Inspection Service, Wildlife Services, as part of the Oral Rabies Vaccination Program in eastern Tennessee, collected serum samples from raccoons. Raccoons were live-trapped using baited single-entry box traps. Animals were anesthetized with an intramuscular injection of ketamine hydrochloride (10 mg/kg) and xylazine (2 mg/kg) and ear-tagged. Approximately 5–6 ml of blood, drawn from the jugular vein, was stored in 8.5-ml Vacutainers on ice packs in the field until serum separation. Sera were separated by centrifugation for 20 min at 2,450 rpm, pipetted into Cryovial, and stored at −20 C. Samples were then shipped to the Tennessee Department of Health, Vector-Borne Disease Lab, Nashville, Tennessee, for serological testing. A lower premolar was extracted and submitted to Matson's Laboratory LLC (Milltown, Montana) for cementum age analysis and tetracycline biomarker determination (Arjo et al., 2008). An annular pattern of "rings" in the tooth is formed as a result of the cyclic nature of cementum growth (Matson, 1981). During the winter, a dark annulus ring is formed, while lightly stained cementum is formed during the spring and summer (Matson, 1981). Matson (1981) developed a standardized cementum aging model for each species. Age is determined by cross-sectioning the tooth and counting the annual cementum growth layers. The presence of tetracycline biomarker is determined by analyzing a thin section of calcified tooth with a fluorescent microscope. Raccoon sex was also determined in the field.

Antigen

Antigen for the IFA assay was prepared as described by Yabsley et al. (2001). Briefly, *T. cruzi* (Brazil strain) epimastigotes were grown in liver infusion tryptose media. Twenty milliliters of a 2- to 3-week-old culture of epimastigotes were collected and centrifuged at 500 *g* for 15 min. The supernatant was removed and the pellet was washed in phosphate-buffered saline (PBS), pH 7.2. The pellet was resuspended in 20 ml of 1%

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TABLE I. Seroprevalence of *Trypanosoma cruzi* in Tennessee counties surveyed from 2005 to 2007.

	2005–2007		2005		2006		2007	
County	# of samples	% positive	# tested	% positive	# tested	% positive	# tested	% positive
Hamilton	408	32.3	154	27.3	130	29.2	124	41.9
Marion	11	63.6	11	63.6	0	N/A*	0	N/A
Meigs	35	20	19	21.1	0	N/A	16	18.8
Hawkins	56	25	0	N/A	36	25	20	25
Washington	41	14.6	0	N/A	0	N/A	41	14.6
Greene	55	32.7	0	N/A	27	29.6	28	35.7
Sullivan	73	19.2	0	N/A	27	25.9	46	15.2
Hamblen	13	30.8	0	N/A	5	20	8	37.5
Unicoi	5	0	0	N/A	5	0	0	N/A
Roane	9	44.4	0	N/A	9	44.4	0	N/A
Total	706	29.3	184	28.8	239	28	283	30.4

* N/A, not applicable.

formalin and allowed to sit at room temperature for 24 hr. The antigen was then stored at 4 C. To prepare slides for the IFA assay, antigen was diluted in PBS until only 20–30 flagellates per field were observed at ×400 magnification. The diluted antigen was then pipetted onto each slide well on 12-well slides (10–20 µl per well), and the slides were dried at 50 C for 30 min. The slides were fixed in acetone for 20 min, allowed to air dry, and stored in a slide box at −20 C for up to 1 mo.

Serology

The IFA assay was used to detect anti–*T. cruzi* antibodies by the method of Camargo (1966), as modified by Yabsley et al. (2001). Positive and negative control sera, in laboratory raised raccoons, were collected from *T. cruzi* experimentally infected and some not, respectively (Roellig et al., 2009). Two-fold serial dilutions of 1:16 to 1:128 for negative controls and 1:32 to 1:256 for positive controls and samples were transferred to a well on a labeled 12-well slide containing *T. cruzi* antigen. Slides were incubated for 30 min at 37 C. After incubation, slides were washed twice in PBS for 5 min and once in distilled water for 5 min. A 1:100 affinity purified goat anti-raccoon immunoglobulin G conjugated with fluorescein isothocyanate (FITC) (Kirkegaard & Perry Laboratories, Gaithersburg, Maryland) prepared in 5% non-fat milk was applied to each slide well. The slides were incubated at 37 C for 30 min in a covered dish to protect the antibody's fluorescence from light exposure during incubation. Slides were washed twice in PBS, then counterstained in 1% Evans Blue for 20 sec, and then washed in distilled water for 5 min. Once slides were dry, a drop of 10% glycerol was applied to each well and a cover slip was added. Slides were examined using an Olympus BX 41 (Olympus, Center Valley, Pennsylvania) fluorescent microscope (excitation filter wavelength BP 460–490), and samples showing strong fluorescence at 1:64 were considered positive. This titer was chosen based on titration experiments performed using positive and negative raccoon control sera. Samples showing weak to indeterminate fluorescence were retested and, if fluorescence was still indeterminate, samples were reported as negative. If epimastigotes appeared dim red and no fluorescence was observed, samples were also reported as negative.

GIS analysis

Spatial analysis was performed using ArcGIS 9.3 with the Spatial Analyst extension. Each sample GPS coordinate was also assigned a land cover characteristic using 2001 land cover data downloaded from the Multi-Resolution Land Characteristics Consortium (MRLC) (http://www.mrlc.gov/nlcd_multizone_map.php). For complete state coverage, zones 11, 12, and 14 were downloaded and clipped with a Tennessee state shape file to obtain land cover data specific to the state. The clipped data were then merged to create 1 statewide land cover database; each land cover characteristic was broken out into individual shape files based on the NLCD 2001 land cover class definitions (http://www.mrlc.gov/nlcd_definitions.php). Land cover class definitions included: developed, low intensity (impervious surfaces account for 20–49% of total cover); medium intensity (impervious surfaces account for 50–79% of the total cover); high intensity (impervious surfaces account for 80–100% of the total cover); grassland/herbaceous; mixed forest; pasture/hay; shrub/scrub; developed, open space (impervious surfaces account for less than 20% of total cover); evergreen forest; deciduous forest; cultivated crops; and barren land (rock/sand/clay). GPS coordinates were designated as rural, suburban, or urban based on their location within each land cover shape file. Habitats were divided into rural and suburban based on population and a shape file was created for each classification. GPS coordinates were identified as falling in rural versus suburban areas (no coordinates fell in urban areas).

Statistics

Data were analyzed using SPSS (Chicago, Illinois). Significance between habitats and land cover class definitions were analyzed using the chi-square test and 1-way analysis of variance (ANOVA). Chi-square and Fisher's exact tests were used to perform statistical comparisons of prevalence rates.

RESULTS

Of the 706 raccoon sera samples tested, 206 (29.2%) were positive for antibodies to *T. cruzi*. Samples with fluorescence at a 1:64 dilution were considered positive for this study, resulting in 206 positive samples for all 3 yr. Seropositive raccoons were detected in 9 of 10 counties sampled and the 3-yr average seroprevalence per county ranged from 14.6% to 63.6% (Table I). In 2005, 53 of 184 (28.8%) samples were positive, with a range of 21.1–63.6% for the 3 counties from which sera were obtained. In 2006, 67 (28%) of the 239 samples were seropositive, with a range of 0–44.4% positive for 7 sampled counties. In 2007, 86 (30.4%) of 283 samples were seropositive, with a range of 14.6–41.9% from 7 counties.

Although there was no significant difference in the overall prevalence during the 3-yr study (Table I), there were differences in prevalence in some individual counties (Fig. 1) between years. More seropositive animals were detected in Hamilton County in 2007 compared to 2005 and 2006 (41.9% vs. 27.3% and 29.2%, respectively, $P = 0.022$), in Greene County in 2007 compared to 2006 (35.7% vs. 29.6%, respectively, $P = 0.631$), and in Hamblen County in 2007 compared to 2006 (37.5% vs. 20%, respectively, $P < 0.001$). Conversely, a decrease in the percentage of positive serum samples from 2006 to 2007 was seen in Sullivan County (25.9% vs. 15.2%, $P = 0.262$) and from 2005 to 2007 in Meigs County (21.1% vs. 18.8%, $P = 0.865$). Little variation was noted from other counties and, although Unicoi County was the only negative county, only 5 animals were sampled in 1 yr.

TABLE II. Odds ratio for habitat, age, and adult risk factors by sex for *T. cruzi* seropositivity in raccoons from east Tennessee.

Risk factor	Description	Odds ratio	95% CI	P value
Habitat	Rural	1.31	1.14–1.52	
	Suburban	0.73	0.61–0.88	<0.001
Age	Greater than 1 year old	1.70	1.49–1.94	
	Less than 1 year old	0.41	0.30–0.55	<0.001
Adult sex	Female	1.76	1.35–2.30	
	Male	0.60	0.48–0.75	<0.001

Raccoons were obtained from rural and suburban areas; 35.1% of raccoons from rural areas were seropositive compared to 23.1% of suburban area raccoons ($P < 0.001$) (Table II). Land cover class was not associated with seropositivity status ($P = 0.441$), even though deciduous forest was the most common site from which raccoons were trapped and the most common site of positive raccoons in rural areas (42.0%). Interestingly, age was positively associated with seropositivity. Raccoons older than 1 yr (adults) were 40.1% seropositive compared to 12.2% of those less than 1 yr (juveniles) ($P < 0.001$, Table II). Among adults, there was a trend of increasing seropositivity with age (Fig. 2).

The prevalence rates were compared by sex in 587 raccoons from which sex information was taken in the field. Of these, 36.16% of females were seropositive compared to 22.4% of males ($P < 0.001$). When 259 juveniles were analyzed only by sex, 16.1% of females were seropositive compared to 15.6% of males ($P = 0.913$). However, among 310 adults analyzed, 52.9% of females were positive compared to 27.7% of males ($P < 0.001$, Table II).

DISCUSSION

The present study provides the first extensive serological examination for antibodies to *T. cruzi* in raccoons from Tennessee. Moreover, the sample size analyzed was larger than in comparable studies in nearby states (Yabsley and Noblet, 2002; Hancock et al., 2005). The percentage of raccoons seropositive for anti–*T. cruzi* antibodies in Tennessee is similar to what has been observed in other serological studies of *T. cruzi* in raccoons in the southeastern United States. In Georgia and Florida, 33% (n = 510) and 68% (n = 108), respectively, of raccoons were seropositive for *T. cruzi* (Brown et al., 2009). In South Carolina, 87 (48%) of 181 raccoons were seropositive, and in Virginia, 154 (33%) of 464 raccoons were seropositive for *T. cruzi* (Yabsley and Noblet, 2002; Hancock et al., 2005). Tennessee had the lowest percentage when compared to these other southeastern states. It is important to note that samples in this study were collected in eastern Tennessee's Cumberland Plateau and the Great Smoky Mountains. The high elevation here results in lower average temperatures compared to the surrounding area. The minimum threshold for *T. cruzi* transmission via the triatomine vector is estimated to be 18 C, and higher temperatures may speed development of the parasite in the insect vector (Intergovernmental Panel on Climate Change, 2001). Since sampling was limited to the cooler region of the state, this could result in an underestimation of the overall prevalence of *T. cruzi* in raccoons in Tennessee. IFA to identify *T. cruzi* infections has high specificity (Camargo, 1966) and is more sensitive than blood smears or culture (Yabsley et al., 2001). Although cross-reaction between *T. cruzi* and other kinetoplastid flagellates, e.g., *Leishmania* spp., can occur, the latter organisms have not been documented in Tennessee wildlife populations. Recent results from studies on the presence of antibodies to *Leishmania* spp. in sera from coyotes (n = 82), redfox (n = 158), and grey fox (n = 51) collected in the other southeastern states found all samples to be negative by IFA (Duprey et al., 2006). Of these wild canids, 3 tested positive in a radioimmunoprecipitation assay (RIPA) for *T. cruzi* (Duprey et

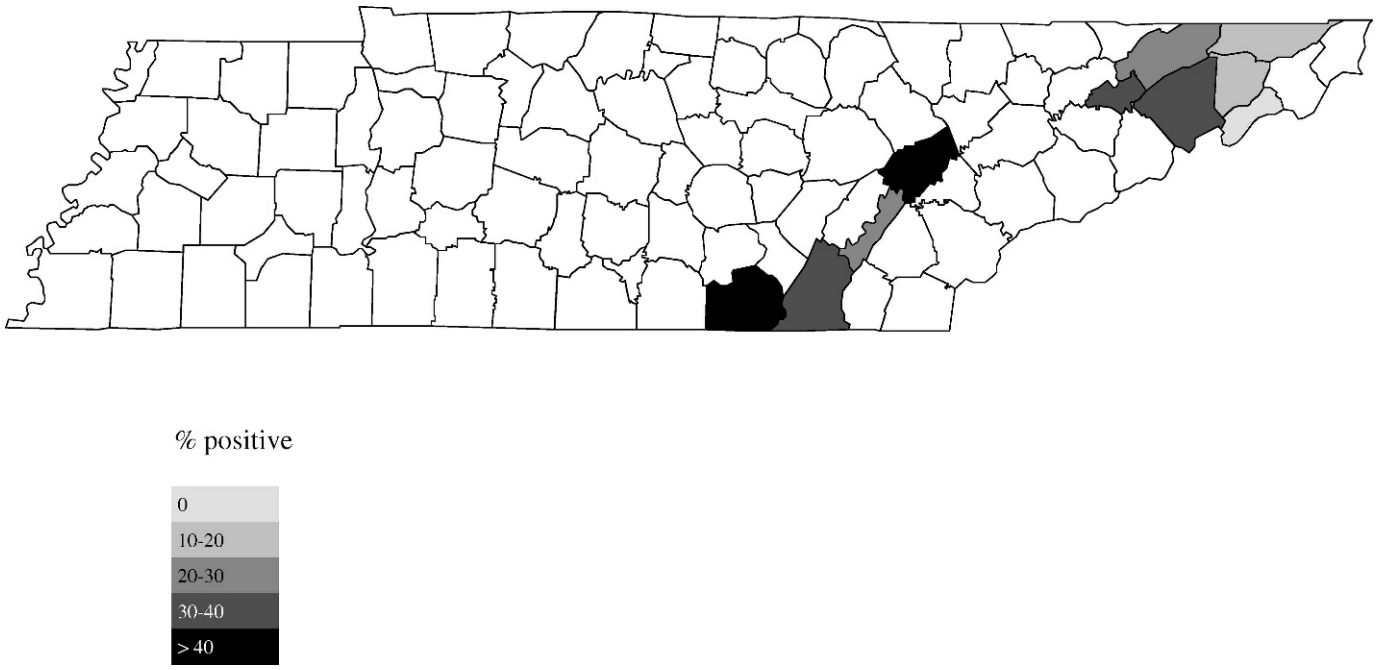

FIGURE 1. Prevalence of antibodies reactive with *T. cruzi* in raccoons from the East Tennessee Ridge and Valley and Blue Ridge Mountains ecoregions of Tennessee.

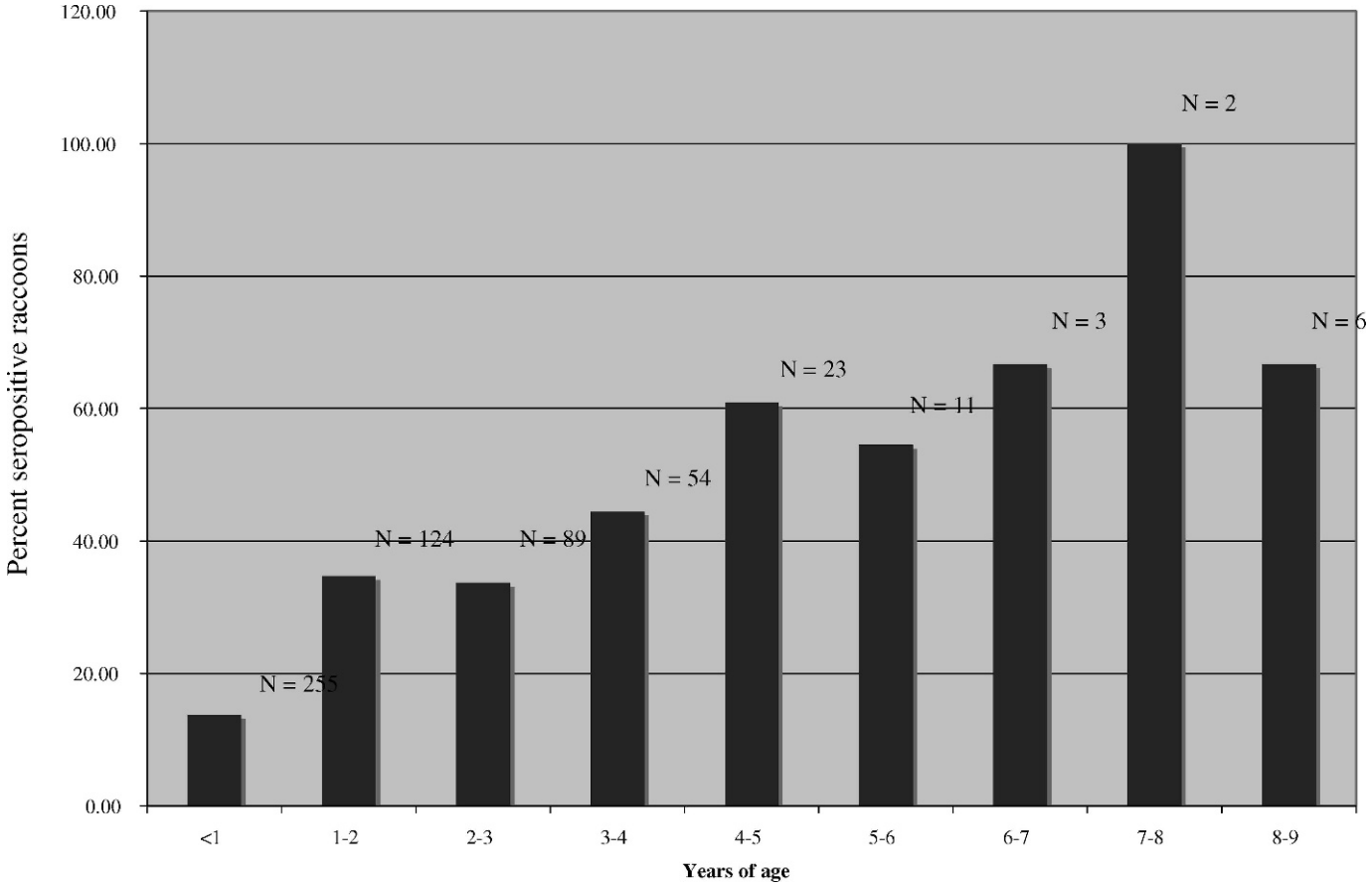

FIGURE 2. Percent of raccoons with *T. cruzi* antibodies by age. Juveniles (<1 yr old, n = 255); Adults (≥1 yr old, n = 312).

al., 2006). Roellig et al. (2008) typed canine and raccoon isolates of *T. cruzi* as being of Lineages I and IIa. When this information is considered in conjunction with the high titer and large sample size used in the present study, it supports the high seroprevalence of *T. cruzi* reported here and makes it unlikely that it would be attributable to cross-reaction with other organisms.

Our results indicate that approximately 30% of the raccoon population of eastern Tennessee has antibodies against *T. cruzi*. Since there is currently no evidence that a mammal can clear an active *T. cruzi* infection, the presence of anti–*T. cruzi* antibodies could be indicative of an active infection in seropositive raccoons. How such a high prevalence of infection might influence raccoon population dynamics can only be speculated. Our observation of age-dependent exposure in raccoons is consistent with age-dependent results in canines, where seroprevalence in dogs increases with age (Burkholder et al., 1980; Castanera et al., 1998; Estrada-Franco et al., 2006). There may be other reasons for lower seroprevalence in raccoon juveniles. First, young animals (<1 yr) are less competent at mounting an antibody response compared to older animals. Second, animals exposed in utero or as neonates may have low or absent serological responses due to the induction of tolerance. Interestingly, a significant difference in seropositivity was seen among female versus male raccoons. Female adult raccoons were almost 100 times more likely to be seropositive than were male adults. This coincides with our recent field observations in Tennessee of kissing bugs being found in tree-holes (data not shown). These tree-holes and other dens are utilized by raccoons and are inhabited by females for litter dens and wintering with kits. Males are not involved in raising young raccoons. This greater exposure of females to tree-holes may put them at greater risk for exposure to kissing bugs infected with *T. cruzi*. It is likely that such den microhabitats are seen in higher densities in rural macrohabitats, partly explaining the higher seroprevalence we observed in rural vs. suburban habitats. Higher levels of exposure also have been observed in female raccoons and opossums in Georgia and Florida (M. Yabsley, pers. obs.).

Parasite pseudocysts are rarely observed in histological studies of wildlife heart tissue. However, in a study of Georgia raccoons, *T. cruzi* pseudocysts were found in the heart tissue of just 1 of 30 raccoons (Pietrzak and Pung, 1998). Other histological studies in Louisiana found pseudocysts in the heart tissue of 6 of 45 opossums, and further examination of the brain, tongue, diaphragm, intercostals muscles, and quadriceps of 30 of the 45 opossums found only 1 pseudocyst in the tongue of a single opossum (Barr et al., 1991). Mild myocarditis was reported in the raccoons and opossums that had pseudocysts present in heart tissue. These findings could indicate that *T. cruzi* that commonly infects raccoons is not pathogenic in this host. Because studies of the pathogenicity of *T. cruzi* in raccoons have utilized small samples of the raccoon population over a brief period of time, it is also possible that these animals were in the early asymptomatic

stages of infection or that, similarly to what is observed in humans, *T. cruzi* induces severe disease in only a small percentage of infected individuals.

The presence of *T. cruzi* in wildlife populations in the U.S. is well established, but to date only 7 autochthonous cases have been reported in humans. There are several factors that could influence the apparently low transmission of *T. cruzi* to humans in the U.S. Thus, more tightly sealed homes may aid in decreasing the potential for the development of a domiciliary cycle. Roofing that attaches directly to the house and the widespread use of screens on windows and doors helps prevent the triatomid vector from entering homes and feeding on the individuals who sleep within. The behavior of the species of triatomine that are present in the U.S. could also play a role in the transmission of *T. cruzi* to humans. Triatomine species in the U.S. have been documented to have delayed defecation times following the acquisition of a blood meal. This reduces the potential for infection since infective metacyclic trypomastigotes will not be deposited on the potential human host (Kagen et al., 1966; Lent and Wygodzinskey, 1979). However, infected *T. sanguisuga* was associated with transmission of *T. cruzi* in both of the recent cases of autochthonous human infection in the U.S. (Herwaldt et al., 2000; Dorn et al., 2007). Climate may also influence *T. cruzi* transmission in the U.S. Since higher temperatures speed pathogen development, the lower average temperatures in the U.S. compared to Central and South American countries could play a role in a lower rate of *T. cruzi* transmission observed in the U.S. Finally, the general lack of awareness among U.S. physicians when considering Chagas' disease as a potential diagnosis when there is not a travel-related association could result in the underreporting of the prevalence of autochthonous *T. cruzi* in humans in the U.S. (Lambert et al., 2008). In combination, these factors may play an important role in the low prevalence of *T. cruzi* in humans in the U.S.

Most human cases of *T. cruzi* are attributable to exposure to metacyclic trypomastigotes via the feces of the triatomid vector. However, this may not be the case in wildlife populations. The foraging behavior of raccoons and opossums, along with their omnivory, may increase their risk for ingesting infected triatomines (Yaeger, 1971; Roellig et al., 2009). This behavior may also contribute to the infection of domestic dogs (Yabsley and Noblet, 2002). In an ongoing study of the seroprevalence of *T. cruzi* in dogs in Tennessee, 6.4% were positive for anti–*T. cruzi* antibodies (A.C. Moncayo, pers. obs.). Because dogs develop persistent parasitemia when infected with *T. cruzi*, they may serve an important role as sentinels of *T. cruzi* infection to humans in domestic cycles due to their ability to infect triatomids (Gurtler et al., 1986, 1991; Kjos et al., 2008). In the human case of *T. cruzi* that occurred in an infant in Tennessee in 1998, the family dog was both IFA and RIPA positive for anti–*T. cruzi* antibodies (Herwaldt et al., 2000). Since infected domestic dogs can facilitate transmission of *T. cruzi* to their owners, a better understanding of the epizootiology of *T. cruzi* in canine populations could help to reduce the risk *T. cruzi* transmission to humans.

The present study demonstrates that *T. cruzi* is well established in portions of the raccoon population in eastern Tennessee. Since raccoon sera from all ecoregions of the state were not available for testing in this study, the observed seroprevalence of 29.2% may not be typical of other parts of the state. Data from neighboring states suggest that it is likely that at least some level of prevalence would be observed if raccoons from every Tennessee ecoregion were tested. The presence of *T. cruzi* in *T. sanguisuga*, a vector of the parasite, has also been reported in Tennessee (Herwaldt et al., 2000; Newsome and McGhee, 2006). Better documentation of the prevalence of *T. cruzi* in animals and insect vectors in Tennessee may aid in lowering transmission risk and directing public health interventions regarding *T. cruzi*.

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